Cell, Vol. 67, 889–899, November 29, 1991, Copyright © 1991 by Cell Press APPENDIX E

bcl-2 Transgene Inhibits T Cell Death and Perturbs Thymic Self-Censorship

Andreas Strasser, Alan W. Harris, and Suzanne Cory
Walter and Eliza Hall Institute of Medical Research
Royal Melbourne Hospital Post Office
Victoria 3050
Australia

Summary

Early death is the fate of most developing T lymphocytes. Because *bcl*-2 can promote cell survival, we tested its impact in mice expressing an Eμ-*bcl*-2 transgene within the T lymphoid compartment. The T cells showed remarkably sustained viability and some spontaneous differentiation in vitro. They also resisted killing by lymphotoxic agents. Although total T cell numbers and the rate of thymic involution were unaltered, the response to immunization was enhanced, consistent with reduced death of activated T cells. No T cells reactive with self-superantigens appeared in the lymph nodes, but an excess was found in the thymus. These observations, together with previous findings on B cells, suggest that modulated *bcl*-2 expression is a determinant of life and death in normal lymphocytes.

Introduction

Cell death plays a critical role in the generation of an effective immune system. In part, this stems from the fact that developing lymphocytes must assemble antigen receptor genes by a recombination mechanism with a high probability of generating out-of-frame joins (reviewed by Tonegawa, 1983; Davis, 1990). As a consequence, most newly generated T and B cells lack a functional antigen receptor and must be purged. Moreover, those that have generated a receptor that happens to recognize a self antigen must be eliminated (or inactivated) in order to prevent the development of autoimmunity.

T lymphocytes are generated and censored in the thymus. Those that successfully assemble functional receptor genes express the heterodimeric (α/β or γ/δ) T cell receptor (TCR) on the cell surface in association with the CD3 protein complex; the variable N-terminal sequences of the TCR polypeptides constitute the antigen-binding site, while signal transduction is mediated via CD3 (for a review, see Marrack and Kappler, 1987). TCRs recognize fragments of antigens bound to molecules of the major histocompatibility complex (MHC) on antigen-presenting cells. This interaction is facilitated by the CD4 and CD8 coreceptors; helper T cells express CD4 and recognize peptide antigens bound to MHC class II molecules, while cytotoxic T cells express CD8 and recognize peptides bound to class I molecules. Only T cells that recognize self MHC molecules in the thymus are selected for potential export to the periphery, but those that can bind to self MHC molecules complexed with self-peptides are deleted by a process termed negative selection (for reviews, see Blackman et al., 1990; von Boehmer and Kisielow, 1990). Selection appears to operate on immature cortical cells that coexpress CD4 and CD8 (Hengartner et al., 1988), a subset comprising 80% of all thymocytes and primarily destined to die within the thymus (Egerton et al., 1990).

The principal form of cell death described for lymphocytes is apoptosis, which is identified morphologically by chromatin condensation and invagination of the nuclear membrane and is usually accompanied by internucleosomal cleavage of cellular DNA (for a review, see Duvall and Wyllie, 1986). Apoptosis is thought to mediate both the clonal deletion of immature T cells (Smith et al., 1989; MacDonald and Lees, 1990; Shi et al., 1991) and the death of antigen-activated mature T cells (Webb et al., l990; Kawabe and Ochi, 1991; MacDonald et al., 1991). The rapid death that can be induced in thymocytes by treatment with glucocorticoids, γ-radiation, phorbol ester, ionomycin, or anti-CD3 antibodies has also been ascribed to apoptosis (Wyllie, 1980; Kizaki et al., 1989; Smith et al., 1989; Shi et al., 1991).

The proto-oncogene *bcl*-2 has recently been implicated as a component of the molecular processes that decide whether a cell lives or dies. *bcl*-2 was discovered as a result of its translocation to the immunoglobulin heavy chain locus in most cases of human follicular center B cell lymphoma (Tsujimoto et al., 1984; Bakhshi et al., 1985; Cleary et al., 1986). This t(14;18) chromosomal translocation spares the coding region of the *bcl*-2 gene but appears to deregulate its expression. The *bcl*-2 gene encodes a cytoplasmic protein (Tsujimoto et al., 1987; Chen-Levy et al., 1989) that seems to be associated with the inner membrane of the mitochondrion (Hockenbery et al., 1990). Insight into its biological function came with the discovery that enforced *bcl*-2 expression delays the death of certain hematopoietic cell lines deprived of growth factors (Vaux et al., 1988). Moreover, transgenic mice expressing a *bcl*-2 gene subjugated to an immunoglobulin enhancer contain a large excess of B lymphocytes with enhanced survival capacity (McDonnell et al., 1989, 1990; Strasser et al., 1990a, 1991), and *bcl*-2 confers a survival advantage on Epstein–Barr virus–infected B cells (Tsujimoto, 1989; Henderson et al., 1991).

In view of the marked effects on B lymphoid cells, it seemed likely that *bcl*-2 would have similar actions in T cells, although early experiments failed to reveal any prolongation of survival in a T cell line infected with a *bcl*-2 retrovirus (Nunez et al., 1990) or in T cells from *bcl*-2-Ig transgenic mice, despite expression of the transgene in the thymus (McDonnell et al., 1989). Here we show that thymic and peripheral T cells from strains of Eμ-*bcl*-2 mice that express the transgene in the T lineage exhibit protracted survival under diverse conditions that decimate normal T cells, including deprivation of growth factors and exposure to ionizing radiation, glucocorticoids, phorbol ester, ionomycin, and sodium azide. Immature thymocytes in Eμ-*bcl*-2 mice are more resistant to killing by anti-CD3

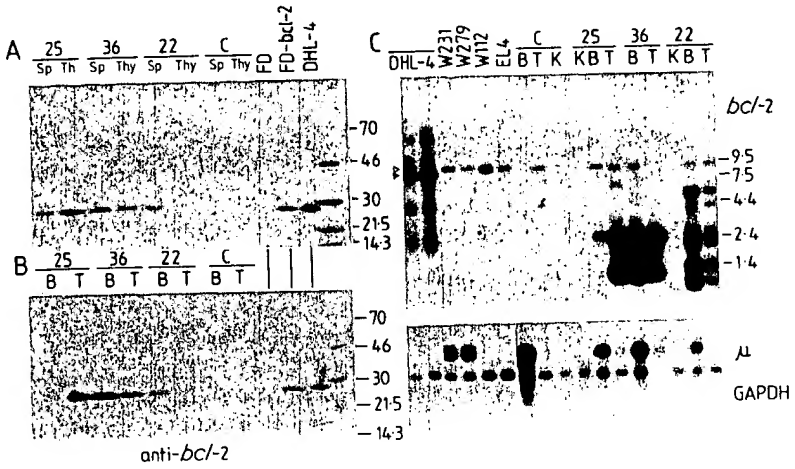

Figure 1. Expression Patterns of the Eμ-*bcl*-2 Transgene

(A and B) Western blot analysis of transgene expression. Nonidet P-40 detergent lysates of thymocytes (Thy), spleen cells (Sp), LPS-activated B lymphoblasts (B), and Con A/IL-2–activated T lymphoblasts (T) prepared from Eμ-*bcl*-2-36, -25, and -22 transgenic mice and nontransgenic controls (C) were fractionated and analyzed for human bcl-2 polypeptide using a monoclonal antibody (Pezzella et al., 1990) as described in Experimental Procedures. Positive controls were the cell line SU-DHL-4 derived from a patient with follicular lymphoma (Chen-Levy et al., 1989) and FD-*bcl*-2, a clone of FDC-P1 cells infected with a retrovirus expressing a human *bcl*-2 cDNA (Vaux et al., 1988). The negative control was uninfected FDC-P1 cells. All samples were the equivalent of 10^6 cells. Coomassie blue staining of duplicate blots showed that the yield of total protein from thymus and spleen was about 3- to 5-fold lower than from the cell lines in (A), and that equivalent amounts of protein were present in all samples analyzed in (B) except for 22B, which had ~50% less. The identity of weak minor bands in preparations from spleen and B lymphoblasts of both normal and transgenic animals is not known. Molecular weight markers are indicated in kilodaltons.

(C) Northern blot analysis of *bcl*-2 transgene expression in SU-DHL-4 cells and in LPS-activated B lymphoblasts (B) and Con A/IL-2–activated T lymphoblasts (T) from spleen cells of Eμ-*bcl*-2-36, -25, -22, or control mice. T lymphoblast preparations from *bcl*-2-22 and -36 contained residual B cells, and B lymphoblasts from *bcl*-2-25 contained residual T cells because of enhanced survival in culture. Roughly 4 μg of polyadenylated RNA was loaded per lane. The filter was hybridized with a probe for the human *bcl*-2 coding region and then washed and rehybridized to a mixture of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and immunoglobulin μ probes. The expected size of the processed transgene transcript is ~2 kb; the smaller RNAs, also seen with other transgenes constructed with the same vector, are probably products of cryptic splice signals. Arrows indicate position of the major transcripts from the endogenous mouse *bcl*-2 gene (open) and the translocated *bcl*-2 gene in DHL-4 (solid). Mouse *bcl*-2 transcript levels in another preparation of B lymphoblasts were comparable to those in T lymphoblasts. Size markers are indicated in kilobases.

antibodies, and cells with high levels of anti-self antigen receptors are apparent in the thymus. Nevertheless, tolerance is not abrogated, since self-reactive cells are not detectable in the mature peripheral T cell pool. The implications of the results for normal *bcl*-2 function are discussed.

Results

To assess the consequences of constitutive *bcl*-2 expression by lymphoid cells, we generated multiple strains of transgenic mice that carry a human *bcl*-2 cDNA under the control of the 5′ *Igh* enhancer (Eμ; Strasser et al., 1990a). Most strains exhibited abnormalities in B lymphoid cells, as described elsewhere (Strasser et al., 1990a, 1991). The Eμ-*bcl*-2-36 strain also displayed T lymphoid abnormalities, while Eμ-*bcl*-2-25 exhibited only T cell effects. These phenotypic differences mirrored the pattern of transgene expression. The protein product of the transgene was detected in both the thymus and spleen of *bcl*-2-25 and *bcl*-2-36 mice by a monoclonal antibody specific for human bcl-2, but only in the spleen of the previously studied *bcl*-2-22 strain, which displays solely B lymphoid abnormalities (Figure 1A). The level of bcl-2 protein detected in T lymphoblasts prepared from *bcl*-2-36 and *bcl*-2-25 spleen cells was comparable to that in SU-DHL-4, a cell line derived from a human follicular lymphoma (Chen-Levy et al., 1989), and in FDC-P1 cells infected with a *bcl*-2 retrovirus (Vaux et al., 1988; Figure 1B). Similarly high levels were apparent in B lymphoblasts of *bcl*-2-36 and *bcl*-2-22 mice (Figure 1B). The level of expression of the transgene is considerably higher than that of the endogenous murine *bcl*-2 gene, as judged by the intensity of the ~9 kb cross-hybridizing band (Figure 1C, open arrow) visible in some, but not all, preparations of RNA from normal and transgenic lymphoblasts and lymphoid cell lines (Figure 1C and data not shown). Strains 25 and 36 were analyzed in detail for perturbations in T cell development and function.

Survival and Differentiation of T Lymphoid Cells In Vitro

Thymocytes expressing the *bcl*-2 transgene survived remarkably well in vitro in the absence of exogenous growth factors (Strasser et al., 1990a). Cell surface marker analysis established that cells of the four major thymic subsets ($CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$) persisted, although the proportion of $CD4^+CD8^+$ cells declined somewhat with time (data not shown). Figure 2 compares the survival of transgenic and normal cells of two thymocyte subsets purified by fluorescence-activated cell sorting prior to plating in simple culture medium. Both cell types displayed enhanced survival, although this was more marked for the $CD4^+CD8^-$ (Figure 2C) than for the major $CD4^+CD8^+$ population (Figure 2A). At 7 days, when 99% of the cells from nontransgenic mice were dead, 10%–35% of transgenic cells remained viable. The persisting cells were small and did not incorporate thymidine (data not shown). Intriguingly, however, some phenotypic changes had eventuated. After 5–7 days in culture, the initially >99% pure $CD4^+CD8^+$ population contained significant numbers of cells lacking one of these markers. The increase in the $CD4^-CD8^+$ population from *bcl*-2-25 cells

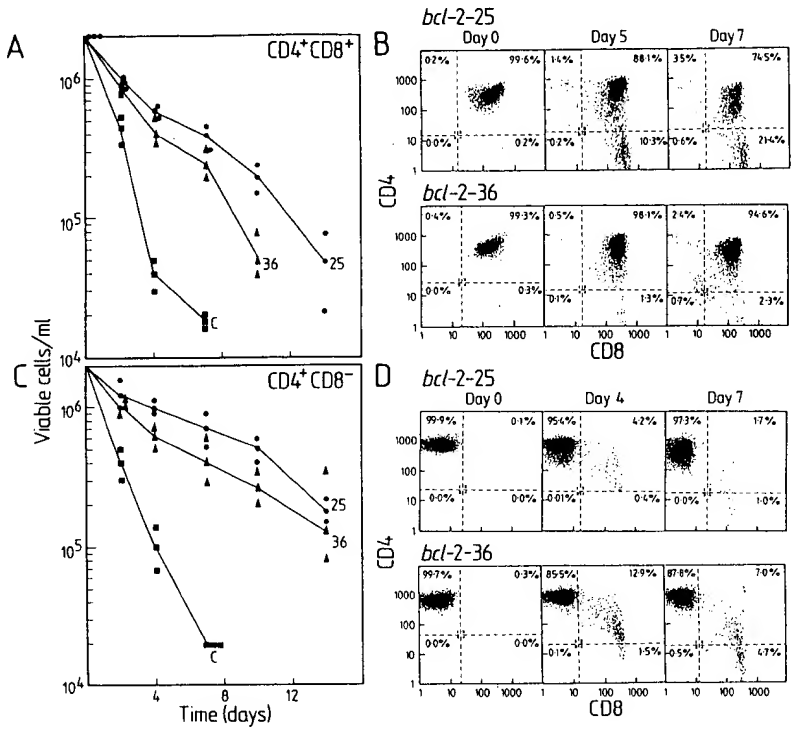

Figure 2. Prolonged Survival and Spontaneous Differentiation of *bcl*-2 Transgenic Thymocyte Subsets

Thymocyte subsets ($CD4^+CD8^+$ and $CD4^+CD8^-$) from 6- to 10-week-old Eμ-*bcl*-2-25 (25) and -36 (36) transgenic mice and nontransgenic (C) littermates were purified by cell sorting.

(A and C) Survival of sorted cells cultured at 2 × 10^6 cells per ml (initial viability ≥95%) in simple tissue culture medium. Concentrations of viable cells were determined in a hemocytometer by trypan blue exclusion.

(B and D) Surface phenotype of surviving cells. On days 0, 4, 5, and 7, cultured cells were harvested, stained with fluorescent antibodies, and analyzed by flow cytometry. Dead cells were excluded from analysis as described in Experimental Procedures.

was particularly noteworthy (Figure 2B), and triple fluorescence analysis demonstrated that this population expressed CD3 at a level as high as that in normal mature $CD4^-CD8^+$ thymocytes (H. Petrie and A. Strasser, unpublished data). In cultures initiated with $CD4^+CD8^-$ cells, both $CD4^+CD8^+$ and $CD4^-CD8^+$ cells appeared (Figure 2D). These results suggest that differentiation can continue spontaneously in vitro when cell viability is sustained by constitutive expression of *bcl*-2 (see Discussion).

Enhanced survival was not confined to thymocytes. Lymph node T cells also survived unusually well in culture (Figure 3A), and the cells remaining after 12 days of incubation included both $CD4^+$ and $CD8^+$ populations (as well as, in the case of *bcl*-2-36, B cells). Transgenic T lymphoblasts activated in vitro by treatment with concanavalin A (Con A) plus interleukin 2 (IL-2) also outlived their normal counterparts when plated in medium containing no stimuli or growth factors (Figure 3B). T lymphoblasts from both transgenic strains exhibited a survival advantage, but for reasons that are unclear, the *bcl*-2-25 cells were consistently less hardy than the *bcl*-2-36 cells, despite a comparable level of transgene-derived bcl-2 protein (see Figure 1B).

The proliferative response of transgenic T cells to Con A plus IL-2 in vitro was no greater than normal and ceased after removal of the stimulus (Figure 4A). The surviving *bcl*-2 T cells all became small (Figure 4B). This was not a result of preferential survival of nonactivated cells, since essentially all cells were large after stimulation with mitogen. Significantly, even after 7 days without growth factors, the small cells could readily be reactivated by Con A (Figure 4A).

Thymocytes Resist Lymphotoxic Agents

The survival of *bcl*-2 transgenic T cells in the absence of growth factors led us to test how well they fared when exposed to agents known to kill normal thymocytes and postulated to act by inducing apoptosis. As shown in Figure 5, thymocytes from both transgenic strains were remarkably resistant in vitro to phorbol myristate acetate (PMA) and to the calcium ionophore ionomycin; they were also less sensitive than normal thymocytes to dexamethasone and γ-radiation. Similar results pertained to purified $CD4^+CD8^+$ cells (data not shown). No cell proliferation was detectable by [^{3}H]thymidine incorporation in the PMA- and ionomycin-treated cultures.

Treatment of normal mice with glucocorticoids or γ-radiation rapidly eliminates cortical ($CD4^+CD8^+$) thymocytes, while mature ($CD4^+CD8^-$ and $CD4^-CD8^+$) thymocytes and peripheral T cells are less sensitive (Scollay et al., 1984). To test the protective efficacy of the *bcl*-2 transgene in vivo, we quantified thymic T cell subsets in mice after they had been injected with hydrocortisone or subjected to γ-irradiation (Figure 6). Less than 1% of normal $CD4^+CD8^+$ cells survived, but 20%–50% of those in transgenic mice persisted after hydrocortisone treatment and up to 20% survived irradiation. Survival was also enhanced for the $CD4^+CD8^-$ and $CD4^-CD8^+$ populations.

Transgenic T cells were also tested for sensitivity to sodium azide, which is reported to kill lymphoid cells by necrosis rather than apoptosis (Shi et al., 1990). Figure 7A shows the effect of sodium azide on the survival of normal and *bcl*-2-25 thymocytes in simple culture medium; Figure 7B displays a similar experiment with Con A/IL-2–activated splenic T cells maintained in IL-2. Both populations

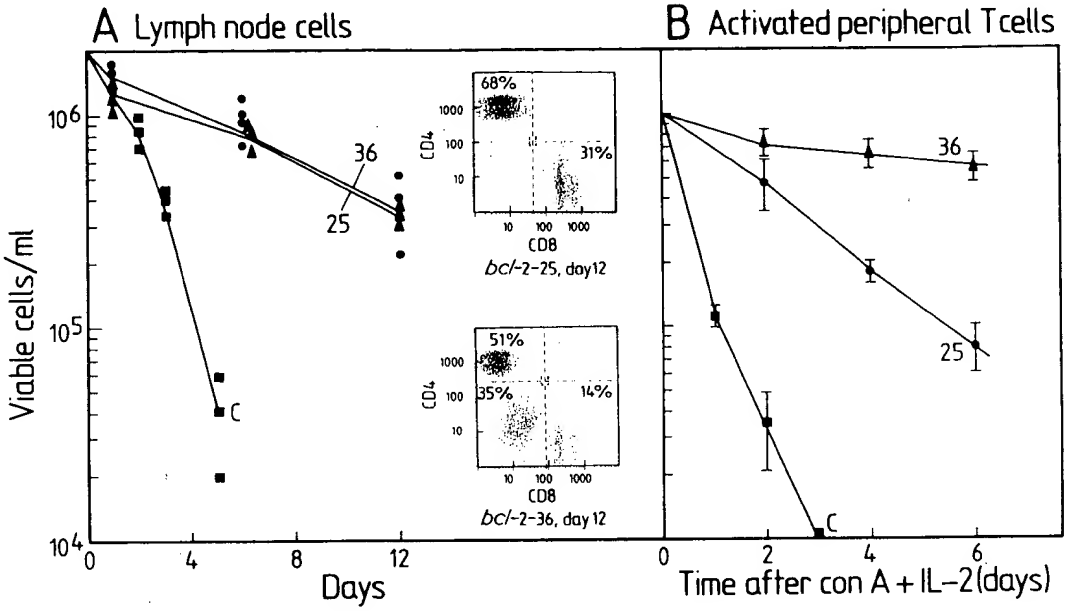

Figure 3. Survival of Resting and Mitogen-Activated Peripheral T Cells

(A) Lymph node cells from Eμ-*bcl*-2-25 (25), -36 (36), and control (C) mice were harvested and plated at 2 × 10^6 cells per ml (viability ≥95%) in simple tissue culture medium. Concentrations of viable cells were determined on the indicated days in a hemocytometer by trypan blue exclusion. On day 12, the surface phenotype of surviving cells was determined by two-color immunofluorescence and flow cytometry. The $CD4^-CD8^-$ population from *bcl*-2-36 mice represents surviving B cells.

(B) Spleen T cells (0.5 × 10^6 cells per ml) were activated by incubation with Con A followed by IL-2 as described in Experimental Procedures, washed three times, and plated at 1 × 10^6 cells per ml (initial viability ≥95%) in simple culture medium. On the indicated days, surviving cells excluding trypan blue were counted. The results are plotted as the mean ± SD of five independent experiments.

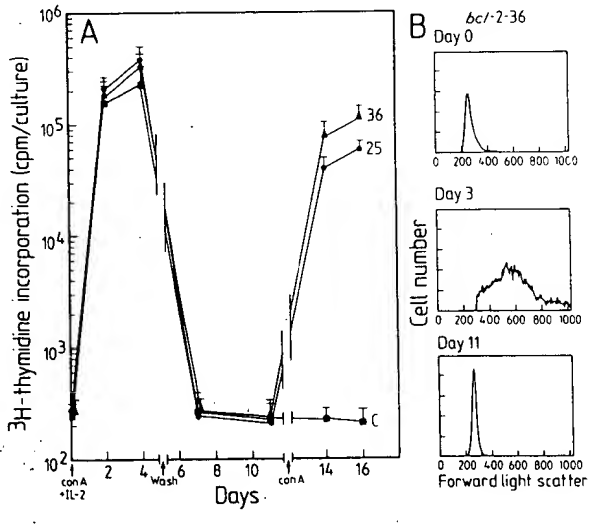

Figure 4. Activation of T Cells In Vitro

(A) Spleen cells (0.5 × 10^6 cells per ml) from Eμ-*bcl*-2-25 (25), -36 (36), and control (C) mice were activated by incubation with Con A (3 days) and then IL-2 (2 days) as described in Experimental Procedures. On day 5, the cells were washed three times and plated at 1 × 10^6 cells per ml in simple culture medium. On day 12, the concentration of viable transgenic cells was readjusted to 2.5 × 10^5 cells per ml and cells were restimulated with Con A. Cell proliferation was measured by [^{3}H]thymidine incorporation for 6 hr on the indicated days. Each data point is the mean of three cultures, and error bars indicate the upper limit of SD.

of transgenic T cells showed enhanced survival compared with nontransgenic cells. T cells expressing *bcl*-2 were not resistant to all cytotoxic agents, however, since transgenic and normal thymocytes were equally sensitive to lysis by anti-Thy-1 antibody plus complement, and transgenic T cells activated with Con A plus IL-2 were killed by cytotoxic T cells as readily as were normal lymphoblasts (A. Strasser, K. Shortman, and M. Hoffmann, unpublished data).

T Cell Homeostasis Appears Normal

Since the transgenic T cells were refractory to diverse cytotoxic agents, it seemed possible that T cell homeostasis would be perturbed. We therefore enumerated T cells in mice of various ages, ranging from 4 days to 1 year. The total number of thymocytes did not differ significantly between transgenic and control mice at any age (Table 1), and neither did the proportions of the major thymocyte subsets (data not shown). Hence, constitutive *bcl*-2 expression during T cell development did not result in thymocyte excess and, perhaps even more surprising, failed to influence the involution of the thymus that accompanies aging. The size and nature of the peripheral T cell pool was

(B) The size of cultured T lymphocytes was measured on the indicated days by forward light scatter flow cytometry. Cultured cells were stained with fluorescein-conjugated anti-Thy-1.2 antibody and propidium iodide, and only $Thy\text{-}1.2^+$ PI^- (viable) cells were analyzed.

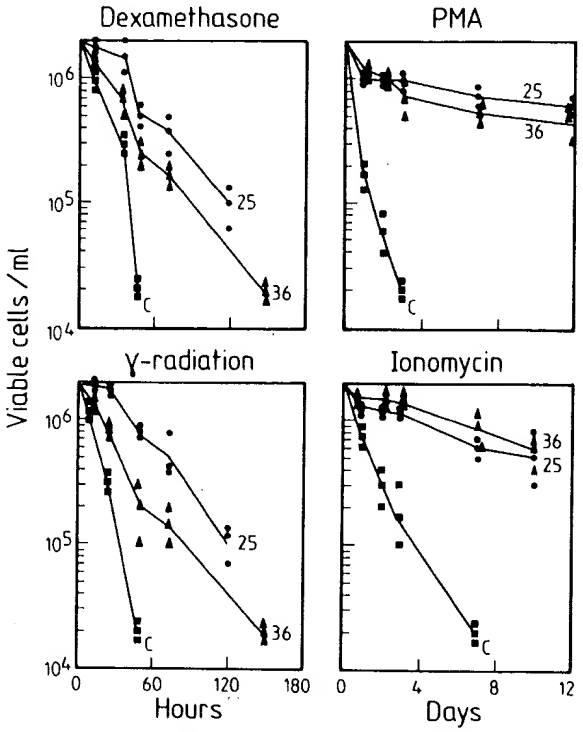

Figure 5. Survival of Thymocytes Treated with Cytotoxic Agents In Vitro

Thymocytes harvested from Eμ-*bcl*-2-25 (25), -36 (36), and control (C) mice were plated at 2 × 10^6 cells per ml (initial viability ≥95%) in simple tissue culture medium in the presence of dexamethasone (1 μM), PMA (5 ng/ml), or ionomycin (1 μg/ml) or after γ-irradiation (10 Gy). At the indicated times, surviving cells were counted in a hemocytometer by trypan blue exclusion.

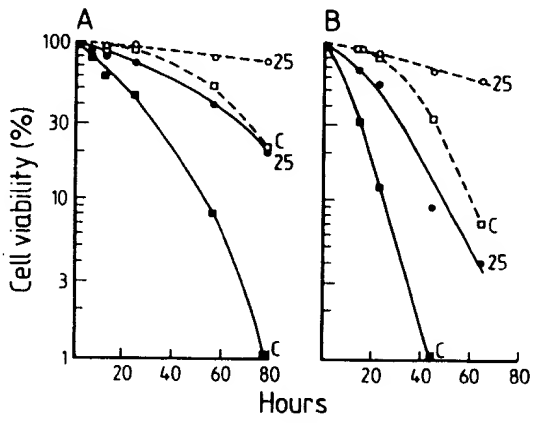

Figure 7. Effect of Sodium Azide on T Cells

(A) Thymocytes harvested from Eμ-*bcl*-2-25 (25) and control (C) mice were plated at 2 × 10^6 cells per ml (initial viability >95%) in simple tissue culture medium in the presence (solid lines) or absence (broken lines) of sodium azide (0.1% w/v).

(B) Spleen cells (1 × 10^6 cells per ml) from Eμ-*bcl*-2 (25) and control (C) mice were activated by incubation with Con A (2 μg/ml for 3 days), then washed three times and replated at 3 × 10^5 cells per ml in medium containing IL-2 (100 U/ml). After incubation for 24 hr, sodium azide was added to 0.1% (w/v). Viable cells were scored at the indicated time points by trypan blue exclusion.

also essentially unaffected. Lymph nodes were normal in size, there was no significant increase in the number of splenic T cells (Table 1), and the ratio of $CD4^+$ to $CD8^+$ cells was normal. Both the thymocytes and the peripheral T cells had normal levels of the surface markers CD3, TCRα/β, Thy-1, and CD5 (data not shown).

Enhanced T Cell Response to Antigen

To explore effects on T cell function, we examined the peripheral T cell response to a so-called superantigen. A much larger proportion of T cells (~1/5–1/50) react with a superantigen than with a conventional antigen (~$1/10^5$) because a superantigen–MHC complex is recognized by particular TCR Vβ elements regardless of their TCR α partner (Pullen et al., 1990). This makes it possible to measure a specific T cell proliferative response to immunization. The superantigen staphylococcal enterotoxin B (SEB) is recognized principally by T cells expressing Vβ8 (White et al., 1989). Splenic T cells bearing Vβ8 were quantified over a 30 day period after injection of SEB. Nontransgenic mice responded with a proliferative burst of both $CD4^+$ and $CD8^+$ cells, and as reported by others (Kawabe and Ochi, 1991; MacDonald et al., 1991), the response peaked at day 2 and then dropped rapidly (Figure 8). In the *bcl*-2 mice, the response peaked on days 2–4 and was 1.5- to 3-fold higher. Since activated T cells seem to die rapidly in vivo (Webb et al., 1990; Kawabe and Ochi, 1991; MacDonald et al., 1991), at least some of this increase could be due

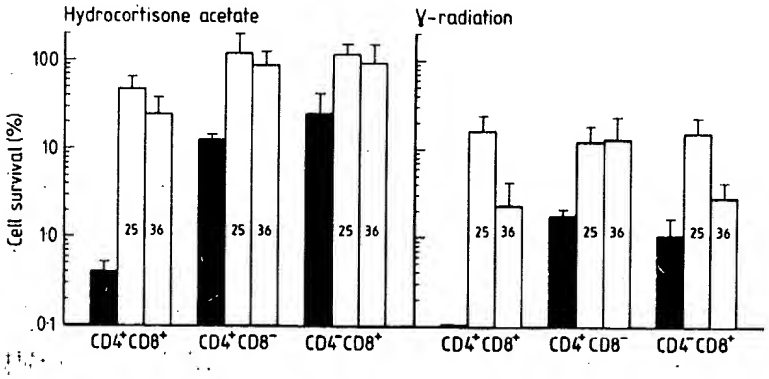

Figure 6. Effects of Corticosteroid Treatment or γ-Irradiation In Vivo

Eμ-*bcl*-2-25 (25), -36 (36), and control (filled bars) mice (3–5 mice per group) were treated by either hydrocortisone acetate injection (1.5 mg per mouse) or γ-irradiation (9 Gy). After 44 hr, the animals were sacrificed, the thymus was removed, and the viable cell content was determined in a hemocytometer by trypan blue exclusion. The percentage of thymic subsets was determined by two-color immunofluorescence staining and flow cytometry. Cell survival is presented as the geometric mean percentage of the viable cells recovered from nontreated mice (3–5 mice per group), with error bars indicating the upper limit of SD.

Table 1. T Cell Content of Thymus and Spleen in Normal and Eμ-*bcl*-2 Mice

	Mouse Strain	Age (weeks)					
		0.5	3	5–6	12–20	27–38	51–55
T cells per thymus ($\times 10^{-7}$)	Control	2.7 ± 0.7	15 ± 4	45 ± 5	14 ± 4	4 ± 1	2.2 ± 1.1
	bcl-2-25	2.8 ± 0.6	17 ± 4	47 ± 3	10 ± 4	7 ± 3	2.3 ± 0.9
	bcl-2-36	3.1 ± 0.9	20 ± 3	48 ± 3	15 ± 6	ND	ND
T cells per spleen ($\times 10^{-6}$)	Control	0.3 ± 0.1	5.8 ± 1.4	37 ± 8	45 ± 5	38 ± 12	ND
	bcl-2-25	0.3 ± 0.0	6.5 ± 3.5	32 ± 3	45 ± 36	42 ± 18	ND
	bcl-2-36	0.4 ± 0.1	10.5 ± 3.7	44 ± 8	73 ± 32	58 ± 14	ND

Total cell numbers were determined by hemocytometer counts, and T cell contents were calculated from flow cytometric analysis of cells bearing CD4 and/or CD8 by two-color immunofluorescence on 10,000 cells per sample. At each indicated age, 2–13 mice (in most cases, 4 or more) of each strain were analyzed, and the results are expressed as the arithmetic mean ± SD.

to enhanced longevity of the T cells. While the Vβ8$^+$ cells in normal mice had declined to basal levels by 7 days, the cells in the transgenic mice declined less, remaining in excess for at least 30 days after immunization. When the T cells from SEB-injected transgenic or control mice were reexposed to SEB in vitro, cells harvested more than 2 days after injection did not respond (data not shown). Thus, like normal superantigen-stimulated T cells (Webb et al., 1990; MacDonald et al., 1991), the *bcl*-2 T cells became refractory to restimulation, a state dubbed "clonal anergy."

Negative Selection Is Affected

Despite the unperturbed total T cell balance sheet, it was important to examine specifically the critical process of deletion of autoreactive T cells. Attempts to mimic this process have used anti-CD3 antibodies, which activate mature T cells but kill the immature thymic cortical CD4$^+$CD8$^+$ cells in vitro (Smith et al., 1989) and in vivo (Shi et al., 1991). In short-term culture, purified CD4$^+$CD8$^+$ thymocytes from *bcl*-2 mice were less sensitive than normal thymocytes to such treatment (data not shown). Furthermore, after injection of anti-CD3 antibody, about 5-fold more CD4$^+$CD8$^+$ thymocytes were recovered from transgenic mice than from normal littermates (Table 2). This effect was CD3-specific, because the recovery of thymocytes from control and *bcl*-2 mice injected with an isotype-matched control antibody was equivalent.

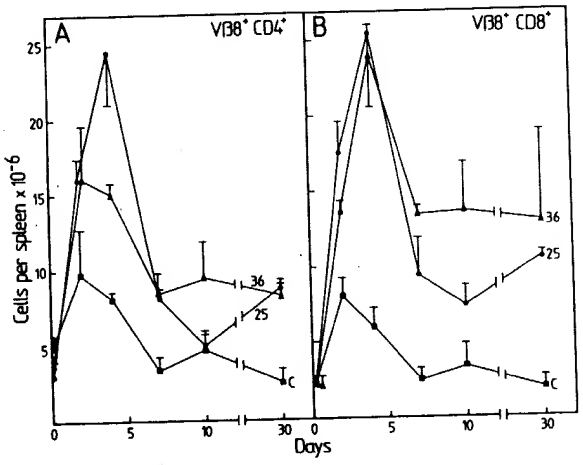

Figure 8. T Cell Response to S. aureus Enterotoxin B In Vivo

Eμ-*bcl*-2-25 × BALB/c (25), Eμ-*bcl*-2-36 × BALB/c (36), and BJF2 × BALB/c control (C) mice were immunized on day 0 with SEB (10 μg intraperitoneally). The BALB/c background was selected for this experiment because responses to SEB are higher in I-E$^+$ mice and have been characterized most thoroughly in a BALB/c context (Kawabe and Ochi, 1991; MacDonald et al., 1991). On the days indicated, 3 animals per group were sacrificed and analyzed for their content of Vβ8$^+$CD4$^+$ (A) and Vβ8$^+$CD8$^+$ (B) spleen cells. Spleens were removed, and the lymphocyte content was determined in a hemocytometer by trypan blue exclusion. Numbers of Vβ8$^+$CD4$^+$ and Vβ8$^+$CD8$^+$ cells were determined by two-color immunofluorescence and flow cytometry, using biotinylated anti-Vβ8 (F23.1) monoclonal antibody plus R-PE–streptavidin- and fluorescein-labeled anti-CD4 or anti-CD8 antibodies (see Experimental Procedures). Data are presented as arithmetic means ± SD of 3 animals per strain per sample time.

These results suggested that *bcl*-2 mice might be defective in negative selection and therefore harbor increased numbers of self-reactive T cells. We tested this possibility directly by measuring the ability of the mice to delete cells responsive to a self-superantigen. Mice expressing the Mls-1^a allele as well as MHC I-E delete their Vβ6- and Vβ8.1-bearing T cells (MacDonald et al., 1988; Kappler et al., 1988), while those expressing Mls-2^a and I-E delete Vβ3 cells (Pullen et al., 1988). The I-E and Mls antigens were introduced into the (C57BL/6 × SJL)F_2 (BJF2) transgenic strains by crosses with DBA/2 (Mls-1^a, Mls-2^a, and I-E^d) and AKR (Mls-1^a and I-E^k) mice to enable us to examine the effect of the transgene on deletion of Mls-reactive T cells in vivo. Table 3 shows the proportion of lymph node cells recognized by Vβ-specific monoclonal antibodies in the relevant strains of mice. Essentially no Vβ3 or Vβ8.1 and very few Vβ6 cells could be detected in the 6- to 9-week-old BJF2 × DBA/2 mice, whether or not they carried the *bcl*-2 transgene. Similarly, both conventional and transgenic BJF2 × AKR mice lacked Vβ6 and Vβ8.1 cells. In case any *bcl*-2 effects might be more apparent during the major phase of thymic expansion, 3-week-old mice were also analyzed. Again, essentially no self-reactive T cells could be detected in the periphery (data not shown).

Although these results indicated that the *bcl*-2 transgene did not abrogate negative selection, it was still possible that it had retarded that process. We reasoned that such an effect might be apparent as an increase in thymic T cells bearing Mls-reactive receptors. As illustrated in Figure 9, although BJF2 × DBA/2 mice contained thymocytes expressing low levels of Vβ3, very few cells had levels as high as those on peripheral T cells in BJF2 mice. Significantly,

Table 2. *bcl*-2 Transgene Expression Interferes with Thymocyte Death Induced In Vivo by Anti-CD3 Antibody

Mice	Antibody Treatment	Viable Thymocytes Recovered	
		Total ($\times 10^{-6}$)	$CD4^+CD8^+$ ($\times 10^{-6}$)
Nontransgenic	Control	4.5 ± 0.5	3.7 ± 0.5
	Anti-CD3	0.5 ± 0.3 (11%)	0.4 ± 0.2 (11%)
bcl-2-25	Control	4.7 ± 0.3	3.6 ± 0.4
	Anti-CD3	2.3 ± 0.2 (49%)	1.7 ± 0.3 (47%)
bcl-2-36	Control	4.8 ± 0.3	4.0 ± 0.5
	Anti-CD3	2.6 ± 1.0 (54%)	2.1 ± 1.0 (53%)

Mice (5–6 weeks old) were injected intraperitoneally with 30 μg of protein A–purified anti-CD3 monoclonal antibody 145-2C11 or with the control, isotype-matched hamster anti-mouse TCRγ/δ monoclonal antibody GL3.1A and analyzed 40 hr later. Thymocytes were counted in a hemocytometer, and the cells in each subset were calculated from flow cytometric analysis of two-color immunofluorescence on 10,000 cells per sample. Between 3 and 6 mice were analyzed per group. Values are the mean ± SD. Cells surviving anti-CD3 treatment are shown in parentheses as a percentage of the viable cells recovered from animals treated with the control antibody.

Table 3. *bcl*-2 Transgene Does Not Prevent Deletion of Mls-Reactive T Cells

Mouse Strain	Haplotype			% of Lymph Node T Cells			
	I-E	Mls-1^a	Mls-2^a	$V\beta3^+$	$V\beta6^+$	$V\beta8.1^+$	$V\beta8.2^+$
BJF2	–	–	–	3.7 ± 0.8	7.2 ± 1.0	2.6 ± 1.1	7.9 ± 2.7
bcl-2-25	–	–	–	3.6 ± 0.4	7.2 ± 1.8	2.3 ± 1.4	8.5 ± 2.7
bcl-2-36	–	–	–	4.1 ± 0.9	8.3 ± 1.7	4.0 ± 1.3	11 ± 2
DBA/2	d	+	+	<0.1	0.6 ± 0.3	<0.1	16 ± 1
BJF2 × DBA/2	d	+	+	<0.1	0.7 ± 0.6	<0.1	12 ± 3
bcl-2-25 × DBA/2	d	+	+	<0.1	1.1 ± 1.0	<0.1	14 ± 2
bcl-2-36 × DBA/2	d	+	+	<0.1	1.4 ± 0.7	<0.1	12 ± 4
AKR	k	+	–	6.5 ± 1.1	1.9 ± 0.8	1.3 ± 0.9	11 ± 2
BJF2 × AKR	k	+	–	6.8 ± 0.5	0.7 ± 0.1	<0.1	9.5 ± 4.1
bcl-2-25 × AKR	k	+	–	6.5 ± 0.7	0.2 ± 0.2	0.5 ± 0.5	7.8 ± 0.8
bcl-2-36 × AKR	k	+	–	6.4 ± 1.3	0.7 ± 0.3	<0.1	15 ± 1

Nontransgenic (C57BL/6 × SJL)F_2 control mice are denoted BJF2. The percentage of Thy-1^+ cells expressing specific Vβ-bearing receptors was determined by two-color immunofluorescence and flow cytometry. The frequency of $V\beta8.1^+$ cells was determined by subtracting the frequency of $F23.2^+$ (anti-Vβ8.2) cells from the frequency of $KJ16^+$ (anti-Vβ8.1 + 2) cells as described by Kappler et al. (1988). Values cited are the mean ± SD for 3–6 mice. Mice homozygous for the SJL haplotype lack $V\beta8^+$ cells as a result of a deletion in the Vβ locus (Behlke et al., 1986), so BJF2 mice with this phenotype were discarded from the analysis.

however, BJF2 × DBA/2 mice expressing the *bcl*-2 transgene had almost as many thymocytes with high levels of Vβ3 expression as control mice, which lacked I-E and Mls-2^a (Figure 9; Table 4). Thus, *bcl*-2 expression afforded some protection to self-reactive thymocytes against negative selection, but not enough to permit such cells to accumulate in the periphery (Figure 9, top; Table 3).

Discussion

This paper documents that *bcl*-2 can enhance T cell survival. A similar conclusion has recently been reached by Korsmeyer and colleagues (personal communication). Thymocytes, peripheral T cells, and activated T cells from Eμ-*bcl*-2 strains expressing the transgene in the T lineage exhibited a remarkable capacity to withstand prolonged culture in vitro in the absence of growth factors. All four major subsets of thymic lymphocytes also resisted killing by γ-radiation, glucocorticoids, ionomycin, and PMA, which are believed to act by inducing apoptosis (Wyllie, 1980; Kizaki et al., 1989; Shi et al., 1991), as well as by sodium azide, which apparently induces necrosis (Shi et al., 1990). The transgenic cells were not, however, resistant to killing by complement or cytotoxic T cells. Thus, *bcl*-2 expression does not interfere with all pathways to cell death.

The resistance of the normally hypersensitive $CD4^+CD8^+$ cell population of the thymus to diverse lymphotoxic agents was particularly striking, because this population contains the self-reactive cells that must be deleted during normal development. Recent evidence suggests that censorship occurs when the TCR/CD3 complex of immature $CD4^+CD8^+$ cells is prematurely engaged by exposure to self-antigen peptides complexed to MHC molecules on thymic stromal cells (reviewed by Blackman et al., 1990; von Boehmer and Kisielow, 1990). Since killing of $CD4^+CD8^+$ cells by exposure to anti-CD3 antibodies has been used as a model for negative selection (Smith et al., 1989; Shi et al., 1991), it was tantalizing to find that the *bcl*-2 transgenic $CD4^+CD8^+$ cells were also resistant to anti-CD3 antibodies, both in vitro and in vivo. Furthermore, whereas conventional mice expressing Mls and MHC I-E antigens lack thymocytes with high levels of Mls/I-E–reactive receptors (Kappler et al., 1987; Ohashi et al., 1990;

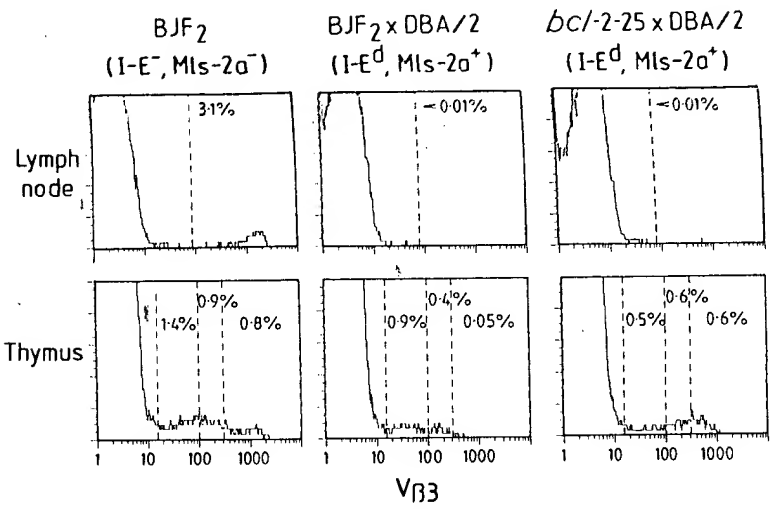

Figure 9. Analysis of Vβ3-Bearing T Cells in Lymph Node and Thymus

Lymph node cells and thymocytes of BJF2, BJF2 × DBA/2, and *bcl*-2-25 × DBA/2 mice were analyzed for the presence of Vβ3-bearing T cells by immunofluorescence staining with biotinylated KJ25 (anti-Vβ3) antibody and R-PE–streptavidin. For lymph node, only T cells (identified by anti-Thy-1-FITC staining) were incorporated into the analysis. From each mouse, 20,000 lymph node T cells and 50,000 thymocytes were analyzed. Data are presented as a frequency histogram with fluorescence intensity on the horizontal axis and relative cell number on the vertical axis. Three markers were set on the lymph node histograms: no. 1 at minimum and no. 3 at maximum fluorescence intensity and no. 2 at channel 80–90, which clearly separates the Vβ3$^+$ from the Vβ3$^-$ population of BJF2 mice. Five markers were set on the thymocyte histograms: no. 1 at minimum and no. 5 at maximum fluorescence intensity, no. 2 was set to include all control stained (R-PE-streptavidin only) thymocytes (channel 17), no. 3 was set arbitrarily (channel 100) to distinguish Vβ3^{lo} from Vβ3^{med} thymocytes, and no. 4 was set at the lower end of the peak of Vβ3$^+$ stained lymph node cells (channel 300) from BJF2 mice.

this work), the *bcl*-2 mice harbored as many of these thymocytes as mice that did not express Mls and I-E (Figure 9; Table 4). Nevertheless, self-tolerance evidently was not compromised in the transgenic mice, since their lymph nodes contained no Mls/I-E–reactive T cells. Our interpretation of these results is that although *bcl*-2 overexpression retards negative selection, extending the lifespan of self-reactive thymocytes sufficiently for them to accumulate in detectable numbers, it does not ultimately save the cells from deletion. Alternatively, intrathymic deletion may be ineffective, but the self-reactive cells may become tolerized in the periphery and therefore not detectable. Although Mls antigens are true self-antigens, some if not all being encoded by endogenous mouse mammary tumor proviruses (Acha-Orbea et al., 1991; Choi et al., 1991), their mode of recognition by TCRs differs from that of conventional antigens. Like other superantigens, they appear to bind outside the groove in MHC molecules utilized by most peptide antigens (Dellabona et al., 1990) and contact only the β chain of the TCR, outside the complementarity-determining region (Pullen et al., 1990). It would therefore be of interest to examine the effect of *bcl*-2 on negative selection in transgenic mice expressing a TCR directed against a more typical peptide antigen.

The *bcl*-2 transgenes used in these T cell studies were identical or similar to those used previously to gauge the effect of *bcl*-2 in the B lineage (McDonnell et al., 1989, 1990; Strasser et al., 1990a, 1991), and it is instructive to compare the results. In both lineages, the ability of cells to survive in the absence of growth factors was manifest at multiple stages of differentiation. Activated lymphoblasts deprived of growth factors appeared to enter a G0 state, but could readily be restimulated to proliferate. T lymphoid homeostasis was essentially unaffected by *bcl*-2, since both the total number of T cells and the proportions of the major subsets were normal in both the thymus and periphery, and thymus cellularity diminished with age at the same rate as in nontransgenic siblings. In contrast, expression of the transgene in the B lymphoid compartment resulted in a large excess of mature B cells and plasma cells. The basis for this difference may be that, unlike many B cells, peripheral T cells are normally very long-lived, and a further boost in their survival capacity would not cause many more to accumulate. Although the

Table 4. *bcl*-2 Transgenic Mice Contain Thymocytes with High Levels of Anti-Self Mls Receptors

Mouse Strain	Haplotype		Vβ3$^+$ Thymocytes (%)		
	I-E	Mls-2^a	Low	Medium	High
BJF1	–	–	1.7 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
BJF2	–	–	1.4 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
bcl-2-25	–	–	1.2 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
DBA/2	d	+	0.8 ± 0.0	0.4 ± 0.1	0.1 ± 0.1
BJF2 × DBA/2	d	+	0.9 ± 0.1	0.4 ± 0.1	0.1 ± 0.0
bcl-2-25 × DBA/2	d	+	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1

The percentage of thymocytes expressing low, medium, or high levels of Vβ3 as determined by flow cytometric analysis of the immunofluorescence intensity distribution from 50,000 cells following staining with biotinylated anti-Vβ3 antibody and R-PE-streptavidin. The fluorescence intensity levels defining each class are specified in Figure 8. Each value is the mean ± SD for 3 mice.

amplitude of both B and T cell responses after antigen injection was greater than normal, the increased antibody response from B cells (Strasser et al., 1991) was probably due both to an increased number of responsive cells and to their longevity, while the enhanced T cell response may be due solely to a decreased death rate of the activated cells. In both responses, the number of activated cells remained higher than normal for several weeks, but this effect was more marked for the antibody-secreting B lymphoid cells than for the SEB-activated ($V\beta8^+$) T cells.

Mice expressing the transgene only in T cells remain healthy for at least a year and no signs of autoimmunity have been detected, consistent with adequate establishment and maintenance of self-tolerance. In contrast, mice expressing the transgene in B lymphoid cells are highly prone to develop an autoimmune disease resembling systemic lupus erythematosus (Strasser et al., 1991). This syndrome was ascribed to the increased number and longevity of the plasma cells, some of which (by chance) secrete immunoglobulins with anti-self reactivity. None of the *bcl*-2 transgenic strains is highly predisposed to tumor development (McDonnell and Korsmeyer, 1991; our unpublished data), although a cryptic predisposition was revealed by introduction of a *myc* transgene (Strasser et al., 1990b).

Since constitutive high *bcl*-2 expression is so effective in promoting cell survival, modulation of *bcl*-2 may be a normal physiological switch governing the life and death of lymphocytes. More specifically, a diminished level of bcl-2 protein may trigger apoptosis, and an elevated level might preserve lymphocytes destined for long life, such as memory B cells generated in the germinal centers of lymphoid tissue and circulating T cells. Consistent with this hypothesis, the level of bcl-2 protein in thymic medullary (mature) cells is considerably higher than that in cortical cells (Pezzella et al., 1990), most of which are doomed to die, and high- and low-expressing B cells can be distinguished in germinal centers (Hockenbery et al., 1991; Liu et al., 1991). Since *bcl*-2 expression is not confined to lymphoid cells, it might also serve as a survival signal in other cell types (Hockenbery et al., 1991).

Regardless of its normal physiological role, *bcl*-2 is a powerful experimental tool. The Eμ-*bcl*-2 mice should be a source of robust T and B cells for production of T cell lines or hybridomas or, indeed, for any immune assay plagued by poor viability or low cloning efficiency. The background of spontaneous lysis in a cytotoxic T cell assay, for example, is markedly reduced if the activated T or B lymphoblasts used as targets are derived from one of the transgenic strains described here (A. Strasser and M. Hoffman, unpublished data). Perhaps the most interesting possibility suggested by our data is that Eμ-*bcl*-2 mice will facilitate analysis of normal lymphoid differentiation. We are currently investigating whether the apparent maturation of Eμ-*bcl*-2 thymocytes in culture reflects survival of a population already committed to make that transition, or whether it is MHC antigen–induced in vitro, as it is in vivo (see reviews by Blackman et al., 1990; von Boehmer and Kisielow, 1990). The latter would be consistent with the observed tendency (Figure 2) of the $CD4^+CD8^+$ population to become $CD4^-CD8^+$ in a milieu in which class I MHC antigens (on neighboring thymocytes) are the predominant cell-bound MHC molecules available.

Experimental Procedures

Mice

The derivation of the Eμ-*bcl*-2 transgenic strains has been described previously (Strasser et al., 1990a, 1991). They were propagated by serially mating heterozygous transgenic mice with normal (C57BL/6JWehi × SJL/JWehi)F_1 hybrid (BJF1) mice. Their genetic background was thus equivalent to BJF2. For certain experiments, transgenic BJF2 mice were bred for one generation with one of the inbred strains DBA/2JWehi (DBA/2), AKR/JWehi (AKR), or BALB/cAnBradleyWehi (BALB/c). Immunization with SEB was by intraperitoneal injection of 10 μg of SEB (Toxin Technology, Sarasota, FL) dissolved in saline. Mice or cell suspensions were γ-irradiated by exposure to a ^{60}Co source at a dose rate of 0.25 Gy/min.

Cell Culture

Cells dispersed from mouse tissues were cultured at 37°C in the high glucose version of Dulbecco's modified Eagle's medium supplemented with 13 μM folic acid, 250 μM L-asparagine, 50 μM 2-mercaptoethanol, 10% fetal bovine serum. Cell viability was determined by trypan blue exclusion and counting in a hemocytometer. T cells were activated in vitro by incubation for 3 days with Con A (Pharmacia, Uppsala, Sweden) at 2 μg/ml, washed three times, and then incubated for an additional 2 days with recombinant mouse IL-2 at 100 U/ml as the culture supernatant from X63/0mIL-2 cells (Karasuyama and Melchers, 1988); B cells were activated by incubation for 3 days with E. coli lipopolysaccharide (Difco, Detroit, MI) at 20 μg/ml. Lymphotoxicity was tested with 5 ng/ml PMA (Sigma, St. Louis, MO), 1 μg/ml ionomycin (Sigma), 1 μM dexamethasone, and 0.1% NaN_3 (w/v). Cell proliferation was determined by radioactivity incorporation after a 6 hr exposure of 100 μl cultures in 96-well microtiter plates to [6-^{3}H]thymidine (1 μCi per culture).

Immunofluorescence, Flow Cytometry, and Cell Sorting

Monoclonal antibodies specific for mouse cell surface antigens were purified from hybridoma culture supernatants by chromatography on protein G– or protein A–Sepharose (Pharmacia) and conjugated to fluorescein isothiocyanate (Molecular Probes, Eugene, OR), R-phycoerythrin (R-PE; Molecular Probes), or biotin (Sigma) by standard methods. The hybridomas used were 53–7.3 anti-CD5, 53–6.7 anti-CD8, and 30-H12 anti-Thy-1.2 (Ledbetter and Herzenberg, 1979); GK-1.5 anti-CD4 (Dialynas et al., 1983); 19-F12 anti-Thy-1.1 (Houston et al., 1980); 145–2C11 anti-CD3 (Leo et al., 1987); KJ25–606.4 anti-TCRVβ3 (Pullen et al., 1988); 44–22–1 anti-Vβ6 (Acha-Orbea et al., 1985); F23.1 anti-Vβ8 and F23.2 anti-Vβ8.2 (Staerz et al., 1985); KJ16–133 anti-Vβ8.1 and 8.2 (Haskins et al., 1984); H57–697.1 anti-TCRα/β (Kubo et al., 1989); GL3–1A anti-TCRγ/δ (Goodman and Lefrancois, 1989); and 2.4G2 anti-Fcγ receptor (Unkeless, 1979).

The conjugated antibodies were titrated, and their specificity was verified on normal lymphocytes and on known Vβ-bearing T hybridoma cell lines (kindly supplied by P. Marrack) and then used routinely at saturating concentrations. Binding of biotinylated antibodies was revealed with R-PE–streptavidin (Southern Biotechnology Associates, Birmingham, AL). To reduce background staining by antibodies binding to cell surface Fc receptors, a saturating concentration of 2.4G2 anti-Fcγ receptor antibody was routinely included in the staining procedure. Multiparameter analysis and sorting of fluorescent antibody-stained cells was done with a FACScan, a FACStar Plus, or a modified FACS II sorter (Becton Dickinson). Dead cells were excluded by staining with propidium iodide (1 μg/ml) and by gating of forward and side scatter of light. Single-color controls were used to set electronic cross-channel compensation for each set of analyses.

bcl-2 RNA and Protein Detection

Transgene mRNA expression was analyzed by Northern blotting of poly(A)$^+$ RNA extracted from cells by standard methods. Nitrocellulose blots were sequentially hybridized with a ^{32}P-labeled human *bcl*-2 cDNA probe corresponding to the 5′ coding region (residues 1–598 in Figure 2 of Cleary et al., 1986) and with a rat glyceraldehyde-3-

phosphate dehydrogenase cDNA probe to monitor RNA loading and transfer efficiency. The polypeptide product of the (human) *bcl*-2 transgene was detected by Western blotting. Lysates of cells from transgenic mice were prepared with Nonidet P-40 (1%) in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (Sigma) (1 mM), clarified by centrifugation, and fractionated by electrophoresis in a reducing 15% polyacrylamide gel. ^{14}C-labeled "rainbow" markers (Amersham, England) were used as molecular weight standards. Proteins were transferred electrophoretically from gels to nitrocellulose membranes, vacant sites were blocked with 1% casein in phosphate buffered saline, and then the washed membranes were reacted with a biotinylated mouse monoclonal anti–human *bcl*-2 antibody, BCL-2 100 (Pezzella et al., 1990), kindly provided by D. Mason. The washed membranes were then reacted with ^{125}I-labeled streptavidin (Amersham) at 10^6 cpm/ml, washed, and autoradiographed with X-ray film.

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